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κ-Opioid receptor agonist inhibition of HIV-1 envelope glycoprotein-mediated membrane fusion and CXCR4 expression on $CD4^+$ lymphocytes

James R. Lokensgard[a,b,*], Genya Gekker[a,b], Phillip K. Peterson[a,b]

[a]*Neuroimmunology Laboratory, Minneapolis Medical Research Foundation, 914 South 8th Street, Bldg. D-3, Minneapolis, MN 55404, USA*
[b]*Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55404, USA*

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Abstract

Our previous studies have shown that the suppressive effect of κ-opioid receptor (KOR) ligand treatment on HIV-1_{AT} (a T-tropic strain) expression in acutely infected $CD4^+$ lymphocytes is time-dependent. This finding implied that the inhibition observed following treatment with KOR agonists such as U50,488 (*trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate) occurs at an early step in the viral replication cycle, perhaps as early as viral entry. In the present study, we examined the hypothesis that U50,488 treatment of $CD4^+$ lymphocytes inhibits HIV-1 envelope (Env) glycoprotein-mediated membrane fusion. We used a vaccinia virus-based assay to measure the effects of U50,488 treatment of $CD4^+$ lymphocytes on HIV-1 IIIB Env glycoprotein-mediated fusogenic activity, based on the cytoplasmic activation of a reporter gene. The results show that U50,488 inhibited Env-mediated cell fusion in a bell-shaped concentration–response manner with suppression ranging between 31 and 98% at concentrations of 10^{-8} and 10^{-10} M ($N = 9$ experiments). U50,488 was also found to inhibit cell fusion when monitored *in situ* with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. Blockade of the inhibitory activity of U50,488 by the KOR antagonist nor-bialtorphimine (nor-BNI) suggested that U50,488 was acting via a KOR-related mechanism. Using flow cytometry, we demonstrated that the chemokine co-receptor CXCR4, but not CD4, is down-regulated as a consequence of KOR activation, with $44.2 \pm 3.5\%$ suppression at 10^{-10} M U50,488. These findings support the hypothesis that KOR-related activation of $CD4^+$ lymphocytes inhibits HIV-1 entry via down-regulation of CXCR4.

Keywords: HIV-1; κ-Opioid receptor; CXCR4; $CD4^+$ lymphocytes; U50,488

1. Introduction

Our laboratory has been investigating the effects of KOR ligands on HIV-1 expression in various types of mononuclear phagocytes for a number of years [1–3]. More recently, we have turned our attention to the effects of these ligands on viral expression in acutely infected $CD4^+$ lymphocytes [4], the primary target for HIV-1. Results generated during recent studies show that the suppressive effect of KOR ligand treatment on HIV-1 expression is both concentration- and time-dependent. In $CD4^+$ lymphocytes, the synthetic KOR ligand U50,488 (*trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate) was found to suppress viral p24 Ag expression by approximately 60% (10^{-7} M); however, no antiviral activity was seen when U50,488 was added to the cells 24 hr after exposure to HIV-1 [4]. This observation implied that the suppressive effects of U50,488 occur at an early step in the viral replication cycle, perhaps as early as viral entry. In the present study, we examined the hypothesis that U50,488 treatment of $CD4^+$ lymphocytes inhibits HIV-1 envelope (Env) glycoprotein-mediated membrane fusion, thereby blocking viral entry into these cells.

HIV-1 entry into target cells involves high-affinity recognition of the cell surface CD4 receptor by the viral Env glycoprotein followed by the interaction of this viral envelope–CD4 complex with an appropriate member of the

* Corresponding author. Tel.: +1-612-347-6849; fax: +1-612-337-7372.
E-mail address: jlokensgard@mmrf.org (J.R. Lokensgard).

Abbreviations: KOR, κ-opioid receptor; GPCR, G protein-coupled receptor; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; FITC, fluorescein isothiocyanate; MOI, multiplicity of infection; CPRG, chlorophenol red-β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; nor-BNI, nor-bialtorphimine.

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seven transmembrane GPCR superfamily for efficient entry. The CXCR4 receptor is used as an entry cofactor by T cell line-tropic (X4) and dual tropic strains of HIV-1 [5,6]. CXCR4 is an α (CXC) chemokine receptor that is expressed in a broad range of cell types including primary $CD4^+$ lymphocytes. Opioid receptors (μ, κ, and δ) are also seven transmembrane GPCRs. Physiologic ligands of seven transmembrane GPCRs have been shown to reduce HIV-1 entry by both blocking and down-regulating the surface expression of these co-receptors [7].

2. Materials and methods

2.1. Reagents

The selective KOR ligand U50,488 was provided by the Pharmacia Upjohn Co., and the κ-selective antagonist nor-BNI was provided by P.S. Portoghese (University of Minnesota). PE-conjugated mouse anti-human CXCR4 antibody (clone 12G5) was obtained from PharMingen, and FITC-conjugated mouse anti-human CD4 antibody was obtained from Coulter.

2.2. Recombinant vaccinia viruses

The following viruses, obtained through the AIDS Research and Reference Reagent Program, were used in this study: vTF7-3, contains the entire bacteriophage T7 RNA polymerase gene expressed under the control of the vaccinia P7.5 promoter [8]; vCB21R-lacZ, contains the *Escherichia coli lacZ* gene under control of the bacteriophage T7 promoter [9]; and vCB-40, contains the HIV-1 *env* gene from HIV-1 IIIB (Lai, BH10) under control of a synthetic vaccinia strong early/late promoter. Viral stocks were grown and tissue culture infectious dose 50 ($TCID_{50}$) titers were determined on MRC5 cells.

2.3. Lymphocyte purification

PBMC were obtained by Ficoll-Hypaque gradient centrifugation (450 *g* for 30 min at room temperature) using Lymphocyte Separation Medium and were activated for 3 days with 4 μg/mL of phytohemaglutinin (PHA) and 10 U/mL of interleukin-2. $CD4^+$ lymphocytes were then isolated from the activated PBMC using Dynabeads® (Dynal) according to the instructions of the manufacturer. $CD4^+$ lymphocytes isolated by this method were found to be >99% pure by fluorescence-activated cell sorting (FACS) analysis.

2.4. Cell fusion assay

The cell fusion assay used in this study was performed as described by Rucker *et al.* [10], with minor modifications. Briefly, adherent HeLa S3 cells (1×10^5/well) were infected with both vCB-40 and vCB21R-lacZ at an MOI = 5 using a 2-hr adsorption followed by overnight incubation in RPMI 1640 medium supplemented with rifampicin (100 μg/mL, Sigma) to inhibit vaccinia virus assembly, minimize the cytopathic effect (CPE), and decrease exogenous virus particles. HeLa S3 cells infected in this way express HIV-1 IIIB Env glycoprotein on their surface (which models the HIV-1 envelope) and also contain the *E. coli lacZ* gene under control of the bacteriophage T7 promoter. Non-adherent $CD4^+$ lymphocytes (1×10^5 cells/well), which are also CXCR4 positive, were infected with vTF7-3 (MOI = 5). This virus expresses the bacteriophage T7 RNA polymerase following infection and overnight incubation. The vaccinia virus infected cells were mixed together and incubated for 6 hr in the presence of ara-C (cytosine β-D-arabinofuranoside, 10 μM, Sigma). ara-C is an inhibitor of viral DNA replication and late-gene expression and is used to inhibit production of new T7 polymerase.

2.5. Reporter gene assays

Following a 6-hr incubation, in which the lymphocytes and HeLa S3 cells were allowed to fuse, the cells were lysed in mammalian protein extraction reagent (MPER, Pierce) for 20 min and subjected to three freeze–thaw cycles. β-Galactosidase expression, as an indicator of cell fusion, in the cell culture lysates was quantified by the CPRG assay (Boehringer Mannheim) as described by Nussbaum *et al.* [11]. Amounts of β-galactosidase activity were determined using a standard curve as described [11]. Syncytia also were stained for β-galactosidase production *in situ* using X-gal (Boehringer Mannheim).

2.6. Flow cytometric analysis of CXCR4

The effect of U50,488 treatment on the cell surface expression of the chemokine receptor CXCR4, which is used as a cofactor for HIV-1 entry into $CD4^+$ T-cells, was examined by flow cytometry. Flow cytometry was performed as previously described [4]. Activated lymphocytes were either left untreated or treated for 30 min with U50,488 prior to staining with CXCR4 antibody (PharMingen) for 30 min on ice. In experiments to determine the specificity of KOR, the T-cells were first treated with nor-BNI (30 min) prior to U50,488 treatment (30 min). At least 10,000 events were analyzed in each sample using a Coulter XL100. The data were evaluated for the percentages of $CD4^+$ lymphocytes expressing CXCR4 using the EPICSr XL software provided by the manufacturer (Coulter Corp.).

3. Results and discussion

Findings from our previous studies showed that U50,488 potently suppresses $HIV\text{-}1_{AT}$ (a T-tropic strain) expression in acutely infected, primary $CD4^+$ T lymphocytes, as

determined by p24 Ag expression, when it is added either 24 or 3 hr prior to infection, as well as simultaneously with the virus, but has no anti-HIV-1 activity when added to the cells 24 hr after infection [4]. This observation suggests that U50,488 may inhibit HIV-1 at an early step in the viral replication cycle, perhaps as early as viral entry. To investigate the effect of U50,488 treatment on HIV-1 Env glycoprotein-mediated membrane fusion of primary $CD4^+$ lymphocytes, we used a vaccinia virus based assay, which quantitates cell fusion-dependent reporter gene activation [7,11].

3.1. Effect of U50,488 treatment on HIV-1 IIIB-mediated membrane fusion

$CD4^+$ lymphocytes and HeLa S3 cells were infected with recombinant vaccinia viruses as described in ''Materials and methods.'' When cells infected in this manner are mixed together, the CD4 and CXCR4 receptors (receptors for HIV) on the lymphocytes interact with the HIV-1 IIIB Env protein on the HeLa S3 cells to mediate membrane fusion between the two cell types. The T7 RNA polymerase in the lymphocyte then interacts with its promoter, carried by the HeLa cell, to stimulate expression of β-galactosidase production in the syncytia.

We first determined the concentration–response of U50,488 treatment on HIV-1 IIIB Env-mediated cell fusion. To determine if U50,488 was acting to inhibit viral entry, we pretreated the vaccinia virus-infected $CD4^+$ lymphocytes for 30 min with a broad range of U50,488 concentrations (10^{-12} to 10^{-6} M) prior to mixing them with vaccinia virus-infected HeLa S3 cells. The U50,488 was not removed from the T-cells prior to mixing them with HeLa S3 cells. The amount of β-galactosidase in the culture was quantified by using the CPRG substrate. In nine separate experiments, a bell-shaped concentration–response was observed with suppression ranging between 31 and 98% at concentrations of 10^{-8} and 10^{-10} M (Fig. 1A).

Next, we investigated if U50,488-mediated inhibition of cell fusion could be detected *in situ* when monitored by X-gal staining, another substrate for β-galactosidase. Vaccinia virus-infected cells were infected and mixed together as described above, and the numbers of fused syncytia, observed as blue cells following staining with X-gal, were counted. When $CD4^+$ lymphocytes and HeLa S3 cells were mixed together in the absence of U50,488, we detected 54.75 ± 4.4 syncytia/5 high power fields (HPF, $N = 4$). In contrast, $CD4^+$ lymphocytes that were treated with U50,488 for 30 min prior to mixing with infected HeLa S3 cells yielded 31.75 ± 1.5 syncytia/5 HPF and 33.5 ± 1.7 syncytia/5 HPF at 10^{-10} and 10^{-8} M U50,488; $P = 0.004$ and 0.0025, respectively, $N = 4$ experiments (Fig. 1B).

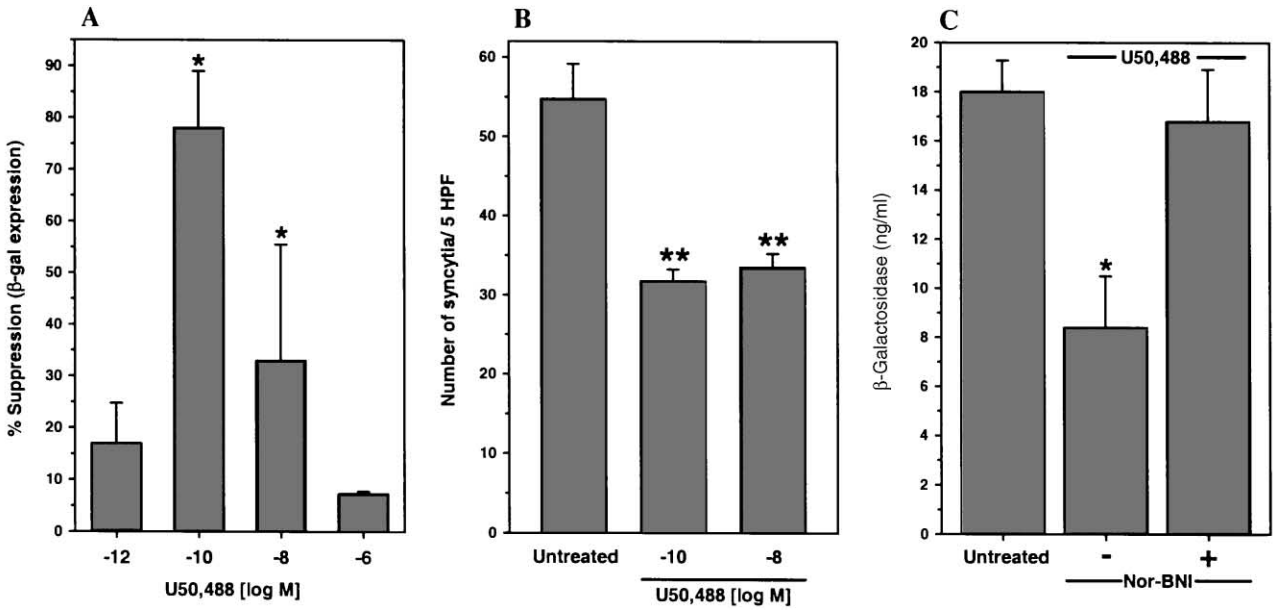

Fig. 1. Effect of U50,488 on HIV-1 IIIB Env-mediated cell fusion with $CD4^+$ lymphocytes. (A) Concentration–response effect of U50,488 on Env-mediated cell fusion. $CD4^+$ lymphocytes were used as partners with HIV-1 IIIB-Env expressing HeLa S3 cells in the presence of a broad range of concentrations of U50,488 (10^{-12} to 10^{-6} M), and fusion was measured using the reporter gene activation assay. Data are expressed as means ± SD of triplicate values and are representative of nine separate experiments with suppression ranging between 31 and 98%. (B) Untreated and U50,488-treated (10^{-8} and 10^{-10} M) $CD4^+$ lymphocytes and HIV-1 IIIB Env-expressing HeLa S3 cells were allowed to fuse for 3 hr, and β-galactosidase expressing syncytia were stained *in situ* using X-gal. Data (means ± SEM) are expressed as the number of syncytia/5 high power fields from four separate experiments. (C) $CD4^+$ lymphocytes were pretreated with the KOR antagonist nor-BNI (10^{-8} M) for 30 min prior to adding U50,488 (10^{-10} M) to determine the involvement of KOR in the suppression of fusion by U50,488. Subsequent cell fusion was analyzed by the colorimetric CPRG assay, and β-galactosidase concentrations (ng/mL) in the lysates were determined from a standard curve. The data shown are means ± SD of triplicate values and are representative of three experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$, versus control, Student's *t*-test.

To investigate whether the inhibitory effect of U50,488 treatment on HIV-1 IIIB-mediated cell fusion was acting through a KOR-related mechanism, we examined the effect of the KOR antagonist nor-BNI on U50,488 inhibition of fusion. When vaccinia virus-infected $CD4^+$ lymphocytes were exposed to nor-BNI (10^{-8} M) for 30 min, prior to adding U50,488 (10^{-10} M) for 30 min, and fused with infected HeLa S3 cells, we found that this KOR antagonist blocked the suppressive effects of U50,488, $N = 3$ different donors (Fig. 1C). This blockade of the inhibitory activity of U50,488 by nor-BNI suggested that U50,488 was acting via a KOR-related mechanism.

3.2. Effect of U50,488 treatment on CXCR4 expression

We have shown recently that 34% of activated primary $CD4^+$ lymphocytes are positive for KOR by flow cytometry [4]. We went on to investigate the possibility that the chemokine co-receptor for T-tropic strains (X4 strains) of HIV-1 (CXCR4) is down-regulated as a consequence of KOR activation using flow cytometry. Calculation of the number of $CD4^+$ lymphocytes that were CXCR4 positive showed that $56.3 \pm 4.8\%$ of the cells bound PE-labeled anti-CXCR4 antibody (Fig. 2, pooled data from four separate experiments). Following a 30-min treatment with 10^{-10} M U50,488, the number of CXCR4 binding lymphocytes was reduced to $31.4 \pm 4.5\%$, resulting in a $44.2 \pm 3.58\%$ suppression of CXCR4 expression by U50,488 treatment (Fig. 2). CD4 was detected on 100% of the $CD4^+$ lymphocytes and was not down-regulated by U50,488 treatment. Although these data strongly suggest an association between the down-regulation of CXCR4 expression and decreased HIV-1 IIIB-mediated cell fusion, the decreased β-galactosidase levels detected following U50,488 treatment may involve a mechanism that is distinct from chemokine receptor expression.

It is unknown how a relatively short (30 min) pretreatment of $CD4^+$ T lymphocytes with U50,488 down-regulates surface expression of CXCR4 in such a rapid manner. Opioid treatment has been shown to inhibit instantaneously chemokine-mediated (but not C5a- or fMLP-mediated) chemotaxis of monkey neutrophils and monocytes [12]. It has also been shown that activation of opioid and chemokine receptors leads to the down-regulation of each other's chemotactic activity through bidirectional heterologous desensitization [13]. Biochemical studies have shown that GPCRs often interact to form homodimers [14]. Most KORs exist as dimers, and it has been shown recently that KORs selectively dimerize with δ- but not μ-opioid receptors [15,16]. Heterodimerization of KORs with other GPCRs such as chemokine receptors (CXCR4) may explain their rapid down-regulation on the cell surface following treatment with U50,488. Experiments to investigate this possible physical interaction and heterodimerization between KORs and chemokine co-receptors for HIV-1 Env are underway. Newly developed ligands that inhibit viral entry into host cells through blocking or down-regulating chemokine co-receptors are promising therapeutic agents for the treatment of HIV-1 infection [17]. Results from the present study suggest that consideration should be given to the development of KOR ligands as HIV-1 entry inhibitors.

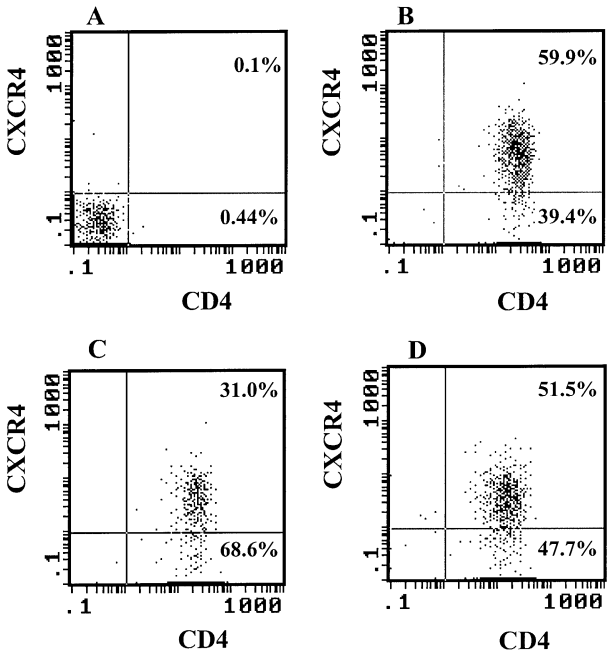

Fig. 2. Effect of U50,488 treatment on CXCR4 expression. Activated $CD4^+$ lymphocytes were treated with U50,488 and double-stained with PE-labeled anti-CXCR4 and FITC-labeled anti-CD4 antibody followed by analysis using flow cytometry. Groups were treated as follows: (A) isotype control antibody; (B) untreated $CD4^+$ lymphocytes; (C) $CD4^+$ lymphocytes treated for 30 min with 10^{-10} M U50,488; and (D) $CD4^+$ lymphocytes exposed to nor-BNI (10^{-8} M) for 30 min followed by treatment with 10^{-10} M U50,488 (30 min). Data are expressed as the percentage of cells positive for each marker and are representative of four separate experiments using $CD4^+$ lymphocytes from different donors.

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